Epstein-Barr virus infection and human malignancies

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Summary. The Epstein-Barr virus (EBV) is a herpes virus which establishes a life-long persistent infection in over 90% of the human adult population world-wide. Based on its association with a variety of lymphoid and epithelial malignancies, EBV has been classified as a group 1 carcinogen by the International Agency for Research on Cancer. In this article we discuss the evidence supporting an aetiological role for EBV in the pathogenesis of human tumours. The biology of EBV infection will be described with special emphasis on viral transforming gene products. A brief survey of EBV-associated tumours is followed by a discussion of specific problems. Evidence is presented which suggests that failures of the EBV-specific immunity may play a role in the pathogenesis of EBV-associated tumours also in patients without clinically manifest immunodeficiencies. Finally, the timing of EBV infection in the pathogenesis of virus-associated malignancies is discussed. There is good evidence that EBV infection precedes expansion of the malignant cell populations in some virus-associated tumours. However, this is clearly not always the case and for some of these tumours there are indications that clonal genetic alterations may occur prior to EBV infection. Thus, whilst there is good evidence to suggest that EBV is a human carcinogen, its precise role(s) in the development of virus-associated human tumours requires clarification.

Keywords: Epstein-Barr virus, infectious mononucleosis, malignant lymphomas, Burkitt lymphoma, Hodgkin's disease, nasopharyngeal carcinoma

Introduction

In the last 10–15 years, the Epstein-Barr virus (EBV) has been detected in the tumour cells of a range of diverse neoplasms. In addition to Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC), the list of EBV-associated tumours now includes Hodgkin's disease (HD), post-transplant lymphoproliferative disorders (PTLD), T-cell non-Hodgkin lymphomas (NHL), gastric carcinomas, possibly breast and hepatocellular carcinomas, and smooth muscle cell-derived tumours in immunodeficient individuals. This has greatly stimulated

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Latency	EBERs*	EBNA1*	EBNA2*	EBNA3 A,B,C	EBNA-LP	LMP1*	LMP2 A*B
I e.g. BL	+	+	–	–	–	–	–
II e.g. HD	+	+	–	–	–	+	+
III e.g. PTLD	+	+	+	+	+	+	+

* viral gene products detectable in formalin-fixed, paraffin-embedded tissue sections by *in situ* hybridization (EBERs) or by immunohistochemistry (all others).

Table 1. Patterns of EBV latency

research into EBV-associated tumours, attracting scientists from different backgrounds into the field. However, the expansion in the number of known EBV-associated tumours has now reached a point where it appears that the virus can be found anywhere if one only looks hard enough. In addition, the EBV association of human tumours shows a range of different patterns. For most of these tumours, the association with EBV is variable depending on factors such as geographical origin of the patient (e.g. BL) or histological subtype (e.g. HD). In some tumours, the virus is present only in a proportion of neoplastic cells (e.g. T-cell lymphomas), raising questions regarding the timing of EBV infection in the pathogenesis of these tumours. Moreover there are at least three different patterns of viral gene expression in EBV-associated tumours. Thus, it is now important to define the role(s) of EBV in the pathogenesis of virus-associated tumours. A preliminary attempt has been made by the International Agency for Research on Cancer (IARC) which, in 1997, classified EBV as a group 1 carcinogen based on its association with BL, HD, PTLD, sinonasal angiocentric T-cell NHL, and NPC (IARC 1997). The purpose of the present article is not to present a comprehensive review of EBV-associated tumours. Rather, we discuss issues relating to the role of EBV in the pathogenesis of human tumours emphasizing the biology of EBV infection, the contribution of viral transforming genes, the role of the immune system and the timing of EBV infection.

Biology of EBV infection

EBV genome

EBV is a member of the lymphocryptovirus genus of the γ herpesvirus family. The structure of EBV is that of other herpesviruses. In the infectious virion, the viral genome is present as a linear, double-stranded, ca.172 kbp DNA molecule. In infected cells, the viral genome persists mainly as an extrachomosomal episome. Episome formation is mediated by a set of 0.5 kbp terminal repetitive (TR) sequences located at either end of the linear molecule and results in a TR region with a variable number of repeats. It is believed that individual infection events lead to episomes which differ in their number of TRs. Thus, analysis of the TR region by Southern blot hybridization can provide evidence as to the clonality of the viral genomes and by implication of the cell population harbouring the virus (Raab-Traub & Flynn 1986).

The viral genome contains potentially over 100 open reading frames (ORFs). The nomenclature of these ORFs is based on a BamHI restriction map of the viral genome (Baer *et al*. 1984). For example, BZLF1 is the first leftward ORF in the BamHI Z fragment (BamHI Z Leftward open reading Frame 1) (IARC 1997).

EBV target cells in vitro

The identification of EBV specific sequences in human tumours of lymphoid or epithelial origin suggests that the virus has the ability to infect cells belonging to very different lineages. However, the efficiency of infection *in vitro* varies dramatically according to the cell type used. EBV readily infects primary resting B-cells from all lymphoid compartments *in vitro* (Rickinson & Kieff 1996). Virus entry includes adhesion to the target cell and entry itself, both phenomena mediated by viral glycoproteins contained in the viral envelope (Rickinson & Kieff 1996). Infection of B lymphocytes has been shown to be mediated by binding of the BLLF1 viral glycoprotein (gp350/220) to the CD21 antigen which also serves as the lymphocyte receptor for the C3d molecule (C3d receptor, CR2) (Fingeroth *et al*. 1984; Nemerow *et al*. 1985; Tanner *et al*. 1987). The BLLF1 glycoprotein is the most abundant glycoprotein in the viral envelope and the major antigen responsible for stimulating the production of neutralizing antibodies *in vivo* (Thorley-Lawson & Geilinger 1980). The BLLF1 glycoprotein not only mediates EBV adsorption to CD21 but binding also induces capping of the receptor and endocytosis of the virus into B lymphocytes. The viral

protein BXLF2 (gp85, gH) is critical for the second step of viral infection, i.e. fusion of the virus with B cells, as has been shown by using a viral recombinant with the neomycin cassette inserted in the BXLF2 open reading frame (Shimizu *et al*. 1996).

Despite isolated reports of successful infection of T lymphocytes, dendritic cells or primary epithelial cells, the efficiency of infection of these cells with EBV has generally been very low, and the experiments difficult to reproduce (Sixbey *et al*. 1983; Watry *et al*. 1991; Lindhout *et al*. 1994). Mature T-lymphocytes from blood or lymphoid tissues appear to be refractory to conventional *in vitro* infection, but a subpopulation of foetal thymocytes expressing the CD21 antigen can be infected and the cells kept in culture for some weeks in the presence of IL2 or thymic presenting cells (Watry *et al*. 1991). These infected thymocytes are reported to express some EBV-encoded antigens but do not show long-term growth (Kelleher *et al*. 1996). In contrast, the T cell leukaemia cell line HSB-2 and the HTLV1 infected cell line MT-2 are readily infected by laboratory strains of EBV (Hedrick *et al*. 1992; Koizumi *et al*. 1992). Interestingly, HSB-2 is CD21 negative, whereas the receptor for EBV entry in MT-2 seems to be an isoform of CD21 which differs from that seen on B-cells.

The binding of EBV to epithelial cells was also thought to result mainly from the interaction of gp350/220 with CD21, and indeed most instances of experimentally induced viral entry into non-B cells has occurred either through ectopic expression of CD21 on these cells or, it has been argued, through very low CD21 levels naturally expressed in the target cell surface (Birkenbach *et al*. 1992; Li *et al*. 1992). This mechanism has also been proposed to mediate EBV infection in the 293 epithelial cell line. However, we have recently shown that a viral EBV mutant lacking gp350/220 still infects the 293 epithelial cell line, as well as B-cells, albeit with a reduced efficiency (Janz *et al*. 2000). These observations therefore point towards the existence of additional viral receptors whose primary function may be the specific infection of non B-cells. In line with this assertion, Wang and Hutt-Fletcher have shown that the gp42 glycoprotein encoded by the viral BZLF2 gene is critical for the infection of B cells but not of epithelial cells (Wang & Hutt-Fletcher 1998). In contrast, the BDLF3 glycoprotein (gp150) was not essential for infection of B cells, and BDLF3 knockout EBV mutants demonstrated an enhanced ability to infect epithelial cells (Borza & Hutt-Fletcher 1998). However, it should be pointed out that the epithelial cells used by this group are stably transfected with the CD21 gene, and therefore represent a rather artificial system.

In summary, the situation is reminiscent of that which has been described for Herpes simplex virus I (HSV1) in which numerous glycoproteins were initially thought to be dispensable for viral infection. In fact some of these glycoproteins were later proved to be critical for infection of primary cells, underlining the need for an appropriate cellular system (Spear *et al*. 2000).

Cocultivation of EBV infected cells with several epithelial cell lines has been shown to be more efficient than infection of the same cells with free viral particles, indicating the potential importance of direct cell-to-cell spread of the infection either via mature or possibly via immature virus particles (Imai *et al*. 1998). This alternative way of infection proved to be remarkably efficient with epithelial cell lines derived from adenocarcinomas of various origins. Whether EBV can be transmitted from one cell type to the other *in vivo* remains to be determined.

EBV-mediated immortalization

Resting normal B-lymphocytes infected with EBV continuously grow under standard culture conditions *in vitro* (Rickinson & Kieff 1996). This unique property has provided a very useful tool to dissect the immortalizing properties of the virus. The lymphoblastoid cell lines (LCL), also called immortalized B-cell lines, represent an excellent model for the post-transplant lymphoproliferative disorders observed in patients with immune deficiencies. However, in many other cases of EBV-associated tumours, the viral proteins identified *in vitro* as central for B-cell immortalization are not expressed, showing that the virus can contribute to cell growth in different ways (Rickinson & Kieff 1996). Cell lines derived from highly malignant Burkitt's lymphomas tumour cells frequently carry the viral genome, and their study has provided important information about the physiology of EBV infection (Rickinson & Kieff 1996).

In EBV infected B-cells, viral multiplication, i.e. lytic replication, is rather unusual. Instead, the viral DNA is replicated by the cellular machinery and is transmitted to daughter cells after cell division, ensuring that the viral genome is faithfully maintained in the infected cell population (Rickinson & Kieff 1996). Only a few of almost 100 genes identified in the EBV genome are actively transcribed in immortalized B-cells, the so-called latent genes. The intracellular localization of these genes has led to the inclusion of their expression products into the EBNA (Epstein-Barr nuclear antigen 1, 2, -LP, 3A, 3B and 3C) and LMP (latent membrane protein 1, 2A and 2B) protein families. Furthermore, two small nonpolyadenylated nuclear RNAs, EBER1 and

EBER2, are abundantly expressed in latent infection (Howe & Steitz 1986). The study of mutant laboratory strains, followed by the construction of purpose made viral recombinants, has led to the conclusion that the expression of the majority of latent genes, if not of all of them, is required for efficient B-cell immortalization (Delecluse & Hammerschmidt, 2000). However, none of these gene products is able to immortalize B-lymphocytes on its own, even if one of them, LMP1, has been shown to transform rodent cells *in vitro* (Wang *et al*. 1985; Baichwal & Sugden 1988). This has led to the concept of latency programs, characterized by distinct patterns of latent gene expression (Table 1). EBV immortalized LCLs express all latent genes (latency type III, Table 1), but in other EBV infected cells the observed pattern of expression can be more restricted, e.g. BL (latency I, Table 1) or HD (latency II, Table 1). In latency III, the EBNAs are transcribed as a long primary transcript originating from one of two promoters located in the BamHI C or W fragments of the viral genome (Cp or Wp). In latencies I and II, EBNA1 alone is transcribed from a different promoter in the BamHI F fragment (Fp) (Schaefer *et al*. 1995; Speck & Strominger 1989).

Viral infection has been shown to be tightly linked to neoplastic transformation in several animal and human tumours. In many cases, viral products directly interfere with the mechanisms that positively or negatively control cell growth. Similarly, EBV infection permanently induces the physiological molecular pathways that lead to B-cell activation, ultimately leading to cell division. The use of conditional systems, in which the expression of one latent gene can be switched on or off, has shown that continuous B-cell proliferation is strictly dependant on viral gene expression. Thus B-cell immortalization is a reversible phenomenon. This is in contrast with common tumour cell lines characterized by the accumulation of irreversible genetic abnormalities. Immortalization, rather than transformation, therefore more accurately describes the immediate consequences of EBV infection in B cells.

EBNA1. This protein is invariably found to be expressed in all EBV infected cells, irrespective of their lineage or differentiation stage. This is not surprising as EBNA1 is crucial for persistence of the viral DNA within the infected cell, and recombinant viruses devoid of EBNA1 lose their ability to immortalize B-cells (Lee *et al*. 1999). EBNA1 binds to several viral DNA domains and in particular to the 20 tandem direct 30 bp repeats present in the *ori*P latent origin of replication as well as to human chromatin (Rawlins *et al*. 1985; Marechal *et al*. 1999). EBNA1 is therefore thought to tether the viral episomes to the chromatin via *ori*P, ensuring their transmission to the cell progeny. EBNA1 was classically thought to be required also for latent viral DNA replication. However, more recent work has cast some doubts on this notion. EBV-derived plasmids can be replicated in the absence of EBNA1 by the cellular machinery but are rapidly eliminated (Aiyar *et al*. 1998). Therefore, EBNA1 is not required for DNA synthesis itself, but allows maintenance of the newly synthetized plasmids. The central part of EBNA1 is characterized by Gly-Ala repeats that play a major role in inhibiting immune recognition. These repeats block processing of the protein by the proteasome and therefore inhibit killing of EBV infected cells by cytotoxic T-cells (Levitskaya *et al*. 1995; Levitskaya *et al*. 1997). More recently, it became clear that EBNA1 elicits a CD4-positive T-cell response, but the contribution of these cells to the anti-EBV response *in vivo* is still unknown (Münz *et al*. 2000). The binding of EBNA1 to *ori*P also leads to an activation of the Cp promoter (Reisman & Sugden 1986). This initiates the transcription of the EBNA common RNA from which the various EBNA proteins are translated. Transgenic mice expressing EBNA1 have been shown to develop tumours, but the actual contribution to immortalization of human cells is still a matter of debate (Wilson *et al*. 1996).

EBNA2, EBNA-LP. The P3HR1 EBV laboratory strain is unable to immortalize B-cells (Miller *et al*. 1974). This strain carries a genomic deletion encompassing the EBNA2 gene and the last two exons of the EBNA-LP gene. Introduction of the EBNA2 and LP genes from the B95.8 viral strain into the P3HR1 genome results in a virus with full immortalizing capacity, demonstrating that EBNA2 is absolutely required for immortalization (Cohen *et al*. 1989; Hammerschmidt & Sugden 1989). EBNA2 has transactivating properties, activating cellular (e.g. CD21, CD23, C-FGR, C-MYC) and viral promoters like the Cp promoter or the LMP1 promoter (Wang *et al*. 1987; Abbot *et al*. 1990; Fåhraeus *et al*. 1990; Knutson 1990; Wang *et al*. 1990; Wang *et al*. 1990; Kaiser *et al*. 1999). EBNA2 is an unusual transactivator as it does not bind directly to DNA but requires an ubiquitous cellular protein, RBP-Jκ, to exert its functions (Henkel *et al*. 1994). RBP-Jκ belongs to the Notch1 receptor signal transduction pathway and binds to specific DNA motifs (GTGGGAA) present in the promoter regions of its target genes (Goodbourn 1995). It then recruits EBNA2 to activate transcription via its acidic transactivating domain. In fact an activated version of the mouse Notch1 can induce the expression of EBNA2 targets

which confirms that EBNA2 makes use of the physiological Notch pathway to activate its targets (Hofelmayr *et al*. 1999; Strobl *et al*. 2000). The PU.1 protein, another DNA binding protein has also been suggested to interact with EBNA2 to activate transcription in EBNA2 responsive promoters (Sjoblom *et al*. 1995). The construction of viral recombinants with deletion of some EBNA2 subdomains has led to a better understanding of the different functions of the protein. Up to now, at least four different subdomains have been defined as essential for immortalization (Cohen & Kieff 1991; Cohen *et al*. 1991; Cohen *et al*. 1992; Tong *et al*. 1994; Yalamanchili *et al*. 1994; Harada *et al*. 1998). Viruses harbouring a deletion of the EBNA-LP gene display a decreased ability to immortalize B-cells (Hammerschmidt & Sugden 1989; Mannick *et al*. 1991). EBNA-LP is currently seen as a coactivator, reinforcing the transactivating properties of EBNA2 (Nitsche *et al*. 1997).

EBNA3A, 3B, 3C. These constitute a related family of genes with limited sequence homology. All members of this family seem to act as transcriptional regulators, with repressing and activating properties. Transfection of an expression plasmid carrying EBNA3C silences the Cp promoter (Radkov *et al*. 1997), but upregulates the LMP1 promoter (Wang *et al*. 1990). EBNA3B transactivates the CD40 and the vimentin promoters (Silins & Sculley 1994). The EBNA3 proteins can bind to RBP-Jκ and therefore inhibit EBNA2 binding to RBP-Jκ (Robertson *et al*. 1995; Robertson *et al*. 1996). The interplay between EBNA2 and the EBNA3 family therefore builds a network that allows precise modulation of the EBV target genes. EBNA3C recruits the human histone deacetylase protein to its targets (Radkov *et al*. 1999). This can account for the silencing properties of the protein, as histone deacetylation represses transcription nonspecifically. Genetic analyses have shown that EBNA3C is absolutely required for B-cell immortalization, as an EBNA3C negative viral recombinant cannot immortalize B-cells (Tomkinson *et al*. 1993). The EBNA3A gene has been reported to be important for the initial process of immortalization, whereas EBNA3B seems dispensable (Tomkinson *et al*. 1993; Kempkes *et al*. 1995).

LMP1. LMP1 is the best studied EBV latent protein. The LMP1 molecules are integral membrane proteins with 6 transmembrane segments that form multimeric aggregates in the plasma membrane resulting in characteristic patches. Transfection of LMP1 in Burkitt's lymphoma cells lines protects against apoptosis via induction of the Bcl2 and A20 antiapoptotic genes (Henderson *et al*. 1991; Laherty *et al*. 1992), and activates the expression of the CD23 cellular gene (Wang *et al*. 1988). Rodent fibroblastic cells transfected with LMP1 display a transformed phenotype, but LMP1 alone cannot transform human cells (Wang *et al*. 1985; Baichwal & Sugden 1988).

The introduction of LMP1 in human cells leads to a permanent activation of several signal transduction cascades through its C-terminal intracytoplasmic domains (see below). LMP1 therefore behaves like a constantly activated receptor that is not dependent on extracellular ligands for its activation (Gires *et al*. 1997). LMP1 shows striking parallels with the CD40 receptor with regard to the signal transduction pathways both molecules utilize (Eliopoulos *et al*. 1996; Eliopoulos *et al*. 1997; Floettmann *et al*. 1998; Kilger *et al*. 1998). LMP1 expression results in the activation of NFkB and therefore of NFkB cellular targets (Eliopoulos *et al*. 1996; Eliopoulos *et al*. 1997; Floettmann *et al*. 1998; Kilger *et al*. 1998). The c-jun N-terminal kinase (JNK)/AP-1 pathway is also a major target of LMP1, and this effect is mediated by the CTAR2 domain (Kieser *et al*. 1997; Eliopoulos & Young 1998). There have been isolated reports of an activation of the JAK-STAT pathway and of the small GTPase cdc42 by LMP1, but their contribution to immortalization is still unknown (Gires *et al*. 1999; Puls *et al*. 1999).

Two subdomains of the C-terminal part of LMP1 have been shown to establish protein–protein interactions with cellular proteins (C-terminal activating regions, CTAR1 and 2) (Huen *et al*. 1995). CTAR1 (amino acids 194–232) has been shown to interact with members of the TRAF (TNF receptor-associated factors) family of proteins (Huen *et al*. 1995). The binding of TRAF2 to CTAR1 seems to be essential for LMP1 function, but TRAF2 can also indirectly bind to CTAR2 (amino acids 351–386) via TRADD (TNF receptor-associated death domain) that allows interaction between both molecules (Kaye *et al*. 1996; Izumi & Kieff 1997). The TRAFs are physiologically recruited by membrane receptors like TNF-R, and this binding leads to a rise in NFkB levels.

The genetic analysis of LMP1 has provided important information about the contribution of the different LMP1 subdomains to immortalization. LMP1 was also shown to be necessary for the continued proliferation and maintenance of the immortalized B cell *in vitro* (Kaye *et al*. 1993; Zimber-Strobl *et al*. 1996; Kilger *et al*. 1998). A further step in the dissection of the function of LMP1 was to generate viral mutants in which only parts of the LMP1 protein were deleted. An EBV which carried a

LMP1 mutant allele in which the first 24 aminoacids from the amino-terminal cytoplasmic domain of LMP1 were deleted kept its ability to immortalize B cells (Izumi *et al*. 1994). In contrast, the first 45 aminoacids of the intracytoplasmic carboxy-terminal part of LMP1 (aa 185–230) were found to be critical for the function of LMP1 whereas deletion of the remaining part of the carboxy-terminus (aa 230–386) could be complemented by cultivating the primary B cells infected with the LMP1 mutant EBV on a feeder cell layer (Kaye *et al*. 1995). The feeder cells were not absolutely required since a high virus titre could obviate the need for it (Kaye *et al*. 1999). However, long-term culture of the infected B lymphocytes proved to be more efficient with the wild-type EBV suggesting that the domain between aa 230–386 of LMP1 plays a crucial role for long-term maintenance of B cells *in vitro* (Kaye *et al*. 1999). This LMP1 domain is likely to comprise additional subdomains with different functions as a virus carrying an LMP1 gene with a deletion from aa 232–351 does not show any difference to wild-type EBV with regard to B cell immortalization (Izumi *et al*. 1999). A more refined analysis of the domain comprising the first 45 aminoacids of the intracytoplasmic carboxy-terminal part of LMP1 provided indirect evidence for a critical role of the TRAF binding site. Using overlapping cosmids, Izumi *et al*. constructed a viral recombinant in which the domain that engages the TRAFs was deleted (aa 185–211) (Izumi & Kieff 1997). After infection of primary B cells with supernatants containing this viral mutant as well as wild-type virus, none out of 412 cell lines contained the mutant virus only, indicating that this particular domain of LMP1 is indispensable for B cell immortalization.

The availability of CD40 -/- mice has allowed a precise evaluation of the respective functions of CD40 and LMP1 (Uchida *et al*. 1999). In fact, introduction of an LMP1 transgene in CD40 -/- mice can only partly rescue the phenotype observed in these animals, showing that the homology in function is limited (Uchida *et al*. 1999). The analyis of LMP1 transgenic mice has provided valuable information about the function of this protein and confirmed that this viral protein interacts intimately with the B-cell activation pathways (Uchida *et al*. 1999). Germinal centre formation is inhibited in these animals, an effect that may be related to the ability of LMP1 and of CD40 to downregulate Bcl6 expression in B-cells. Bcl6 is required for germinal centre formation, as has been shown by the study of Bcl6 knock out mice (Cattoretti *et al*. 1995). The observation that the LMP1 transgenic CD40 -/- mice can produce high affinity antibodies in the absence of germinal centres is more puzzling. Whether LMP1 can directly interfere with the hypermutation machinery or whether LMP1 unmasks physiological extrafollicular hypermutations is not known.

LMP2A and B. These proteins are encoded by the same viral gene, with the exception of the first exon that is unique to LMP2A. It has been speculated therefore that LMP2B could therefore act as a negative regulator of LMP2A function (Longnecker & Miller 1996). Like LMP1, LMP2A is an integral membrane protein with 12 transmembrane domains that forms patches and interferes with the cell signalling pathways. The intracytoplasmic tail contains an ITAM (immunoreceptor tyrosine based activation motif) domain that can recruit the src and the syk families of kinases (Longnecker & Miller 1996). These protein tyrosine kinases are physiologically involved in signal transduction from the B-cell receptor. Binding of the tyrosine kinases to the phosphorylated LMP2A ITAM therefore suppresses the activating signal normally generated by the B-cell receptor (Longnecker & Miller 1996). Akata, an EBV positive Burkitt's lymphoma cell line, can be induced to produce virions by addition of anti-Ig. This suggests that crosslinking of the B-cell receptor leads to reactivation of the viral lytic cycle. The ability of LMP2A to repress activation signals mediated by the BCR can explain a repressive effect on lytic replication (Longnecker & Miller 1996).

Mice carrying an LMP2A transgene show an increase in bone marrow and peripheral B lymphocytes that did not undergo functional Ig rearrangements (Caldwell *et al*. 1998). It is tempting to speculate that the ability of LMP2A to generate signals usually transmitted by functional Ig receptors allowed survival of these B-cells. It has been reported that both LMP2A and LMP2B do not contribute to the immortalization process of mature resting B cells *in vitro* (Kim & Yates 1993; Longnecker & Miller 1993) although a different report exists (Brielmeier *et al*. 1996) and functional data argue for an essential role in the infection of differentiating B cells (Caldwell *et al*. 1998; Caldwell *et al*. 2000). Even more detailed mutations of LMP2A in the context of the complete EBV genome support a role for LMP2A in the interference with immunoglobulin signal transduction (Fruehling *et al*. 1996; Fruehling & Longnecker 1997; Fruehling *et al*. 1998; Swart *et al*. 1999). Recently, it has also been shown that interaction of LMP2A with extracellular matrix proteins may trigger intracellular signalling in epithelial cells involving Csk, a negative regulator of Src kinase (Scholle *et al*. 1999).

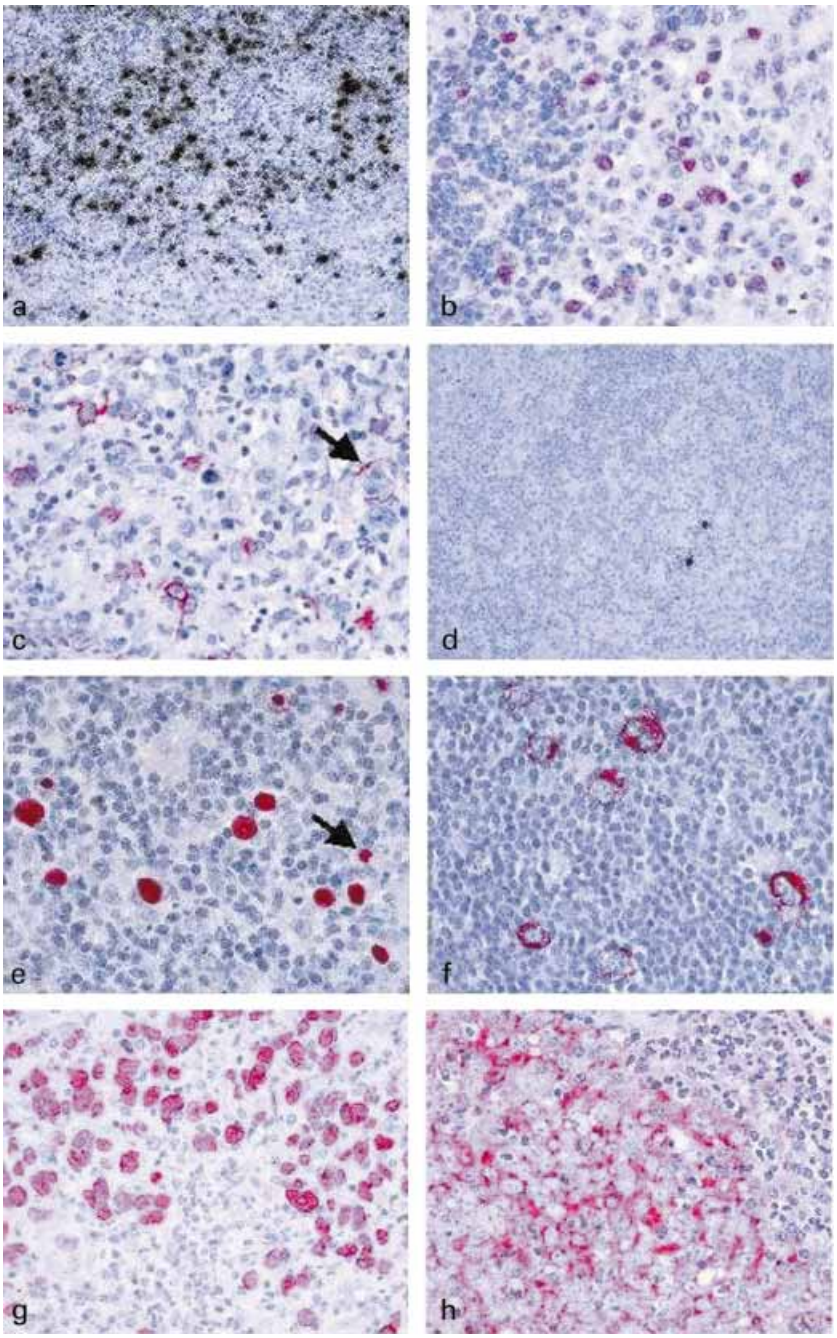

Figure 1. *In situ* hybridization with ^{35}S-labelled EBER-specific probes reveals numerous EBV-infected cells in a tonsil from a patient with acute infectious mononucleosis (a, black grains). Immunohistochemical analysis of a tonsil from an infectious mononucleosis patient reveals expression of EBNA2 (b, red labelling) and of LMP1 (c, red labelling; note a LMP1-positive Reed-Sternberg-like cell, arrow) in many cells. Only rare EBV-positive cells are detectable in a tonsil from a chronic virus carrier (d, black grains). EBER-specific *in situ* hybridization of a case of Hodgkin's disease using nonradioactive probes reveals the presence of the virus in Hodgkin and Reed-Sternberg cells (e, red labelling; note isolated EBV-positive small reactive lymphocytes, arrow). EBV-positive Hodgkin and Reed-Sternberg cells also express LMP1 (f, red labelling). EBV is detected in the neoplastic cells of an undifferentiated nasopharyngeal carcinoma using nonradioactive EBER-specific *in situ* hybridization (g, red labelling). In the same case, the tumour cells also express LMP1 (h, red labelling).

Primary and persistent EBV infection in vivo

Primary EBV infection occurs usually in childhood and then is asymptomatic in most cases (Evans 1972). In most industrialized countries, primary infection is delayed into adolescence or early adulthood and then may cause a self-limiting lymphoproliferative disorder, infectious mononucleosis (IM) (Evans 1972). Most of what is known of primary EBV infection is derived from studies of IM patients and is based on the assumption that IM is representative also of asymptomatic primary infection. IM is characterized by a proliferation of EBV-infected, activated B-lymphoid blasts in the paracortex of tonsillar lymphoid tissue (Figure 1a) (Niedobitek *et al.* 1989). In IM, a latency III pattern of viral gene products is detectable in RNA extracts (Figure 1b, c) (Tierney

et al. 1994). However, analysis at the single cell level reveals some heterogeneity with cells displaying latencies I, II, and III and cells with an EBNA2$^+$/LMP1$^-$ phenotype being detectable (Niedobitek *et al*. 1997). A proportion of EBV-infected B-cells in IM shows evidence of plasmacytoid differentiation, and these cells may support virus replication (Niedobitek *et al*. 1997). EBV-infected cells of other lineages, e.g. epithelial cells or T-cells, are rarely if ever detectable in IM (Karajannis *et al*. 1997; Niedobitek *et al*. 1997; Niedobitek *et al*. 2000).

The proliferation of EBV-infected B-cells in IM elicits a vigorous cytotoxic T-lymphocyte (CTL) response which is directed against latent and lytic viral proteins (Rickinson & Kieff 1996; Steven *et al*. 1997). CTLs specific for all EBNAs and LMPs with the exception of EBNA1 have been identified (IARC 1997). Recent work has also identified a consistent response of CD4$^+$ T-cells to EBNA1 presented by dendritic cells (Münz *et al*. 2000). This T-cell response allows the transition from primary infection into asymptomatic life-long persistent infection. In persistent EBV infection, a small number of EBV-carrying B-cells is detectable in peripheral blood and lymphoid as well as other tissues (Figure 1d) (Niedobitek *et al*. 1992; Hubscher *et al*. 1994; Khan *et al*. 1996). The number of these cells varies between individuals but appears to be constant within any given person (Khan *et al*. 1996). Present evidence suggests that EBV persists in the B-cell system but the exact mechanisms mediating viral persistence are uncertain (Niedobitek & Young 1994; Thorley-Lawson *et al*. 1996). Thorley-Lawson's group has presented evidence implicating memory B-cells as the major site of EBV persistence and it is possible that EBV utilizes germinal centre reactions to gain access to the memory B-cell pool (Niedobitek *et al*. 1992; Thorley-Lawson *et al*. 1996; Babcock *et al*. 1998). Alternatively, EBV may directly infect memory B-cells as demonstrated for infectious mononucleosis (Kurth *et al*. 2000). Persistent EBV infection of B-cells is characterized by a restricted pattern of viral latent gene expression, but exactly which genes are expressed is a matter of debate. Using RT-PCR studies, EBNA1 and LMP2A transcripts have been demonstrated in peripheral blood lymphocytes (Tierney *et al*. 1994). Studying a series of tonsils, Babcock reported the detection of a latency III pattern in naive B-cells and a latency II pattern in germinal centre and memory B-cells (Babcock *et al*. 2000). Peripheral blood memory B-cells showed an even more restricted expression of latent genes with only LMP2 but remarkably no EBNA1 detectable (Babcock *et al*. 2000). There are, however, two concerns regarding this study. Firstly, the tonsils studied were apparently poorly characterized. There was no information available regarding the history of the patients or the histopathological changes observed in these specimens. Secondly, the authors used a sensitive RT-PCR method and thus, the relevance of the transcripts detected remains uncertain. Using immunohistochemical techniques, we have detected only rare EBNA2-positive and no LMP1 positive cells in tonsils from chronic virus carriers (Meru *et al*. in press).

EBV can clearly infect other cell lineages as illustrated by the detection of the virus in a variety of human tumours derived from, e.g. T-cells, epithelial cells and even smooth muscle cells. Thus, it is possible that, in addition to B-cells, cells of other lineages contribute to EBV persistence and replication. Alternatively, infection of such cell types might be regarded as accidental. In particular, the relevance of oropharyngeal epithelial cells for primary and persistent EBV infection requires re-evaluation, in particular because oral hairy leukoplakia, an epithelial lesion of the tongue, remains the best example for EBV replication *in vivo* (Niedobitek *et al*. 1991a).

EBV-associated tumours: a brief survey

EBV-associated lymphomas

Burkitt's lymphoma (BL). BL is endemic in equatorial regions of Africa and Papua-Guinea where it is a common childhood cancer (Burkitt *et al*. 1965). In Western countries, BL occurs as a sporadic tumour. BL is a B-cell non-Hodgkin lymphoma (NHL) with an origin from germinal centre B-cells (see below) (Gregory *et al*. 1987). All BL cases characteristically carry a chromosomal translocation, t(8; 14), t(8; 2), or t(8; 22), placing the *c-myc* oncogene under the control of an immunoglobulin gene (Klein 1979). This results in an inappropriate overexpression of *c-myc*. By contrast, endemic and sporadic BL differ with respect to EBV-association: virtually all endemic BLs carry the virus while EBV is detectable only in up to 30% of sporadic cases (Zur Hausen *et al*. 1970; Hummel *et al*. 1995). In EBV-associated BL, the viral genome is present as a monoclonal episome and expression of the viral latent genes is restricted to the EBERs and EBNA1 (latency I) (Neri *et al*. 1991; Raab-Traub & Flynn 1986; Rowe *et al*. 1987; Niedobitek *et al*. 1995).

Hodgkin's disease (HD). HD is characterized histologically by the presence of Hodgkin and Reed-Sternberg (HRS) cells admixed with abundant lymphocytes and

other reactive cells. Four histological types of HD have been defined based on the morphology of the HRS cells and the composition of the background infiltrate: lymphocyte predominant (HDlp), nodular sclerosing (HDns), mixed cellularity (HDmc), and lymphocyte depleted (HDld) Hodgkin's disease (Harris *et al*. 1994). HDlp has been recognized as a separate entity representing a B-cell lymphoma derived from germinal centre B-cells and behaving clinically differently from the other three forms of HD which collectively are called classical HD (Harris *et al*. 1994). Most cases of classical HD are also derived from B-cells (see below) although a small proportion of T-cell derived HD have been described (Kanzler *et al*. 1996; Müschen *et al*. 2000).

In western countries, EBV is detectable in the HRS cells of 20–50% of HD cases while in developing countries up to 100% of cases may be associated with the virus (Ambinder *et al*. 1993; Herbst *et al*. 1993). Childhood HD appears to be more frequently associated with EBV than HD occurring in young adults (Armstrong *et al*. 1998). Moreover, the virus is commonly detected in HDmc cases, while HDns and in particular HDlp are less frequently EBV-associated (Herbst *et al*. 1993). Analysis of the EBV terminal repeats has revealed monoclonal viral episomes in HD (Anagnostopoulos *et al*. 1989). In all EBV-associated HD cases, LMP1 and LMP2A are expressed in addition to the EBERs and EBNA1 (Figure 1e,f; latency II) (Herbst *et al*. 1991; Deacon *et al*. 1993; Grässer *et al*. 1994; Niedobitek *et al*. 1997).

Immunosuppression-related lymphoproliferations. Immunosuppressed transplant patients are at an increased risk of developing lymphoproliferative disorders (post-transplant lymphoproliferative disorders, PTLD). The vast majority of these cases are B-cell-derived and EBV-associated. PTLD comprise a spectrum of diseases ranging from polyclonal polymorphic lesions to frankly malignant monomorphic and monoclonal lymphomas which are morphologically indistinguishable from lymphomas occurring in nonimmunosuppressed individuals (Craig *et al*. 1993). Polymorphic PTLDs tend to display a type III latency although there is usually a degree of variability at the single cell level (Oudejans *et al*. 1995). In monoclonal lymphomas, the expression of viral latent genes is generally more restricted, conforming to latency patterns I or II (Niedobitek *et al*. 1997a). Patients with acquired immunodeficiency syndrome (AIDS) also have an increased risk of developing malignant lymphomas. It has been estimated that at least 1–4% of AIDS patients suffer from this disease (IARC 1997). A large proportion of these lymphomas are primary CNS lymphomas which account for 20–60% of cases (IARC 1997). Morphologically, AIDS-related primary CNS lymphomas represent diffuse large B-cell lymphomas. The vast majority of these cases are EBV-positive with variable expression of LMP1 (MacMahon *et al*. 1991). Systemic AIDS-related NHL fall into two groups: BL and diffuse large B-cell NHL (Hamilton-Dutoit *et al*. 1991). The latter are mostly EBV-positive and display latencies II or III (Hamilton-Dutoit *et al*. 1993). While most of these lymphomas are monoclonal, rare polyclonal cases have been reported suggesting a similar pathogenesis as for PTLD (Gaidano & Dalla-Favera 1995). AIDS-related BL, by contrast, appear to be less frequently EBV-associated and then usually express a type I latency. Thus, these lymphomas may be pathogenetically similar to sporadic BL (Hamilton-Dutoit *et al*. 1993; IARC 1997).

Other B-cell NHL. In spite of its B-cell tropism, EBV is only infrequently detected in conventional B-cell lymphomas. Moreover, if present, the virus is often detectable only in a proportion of neoplastic cells (Hummel *et al*. 1995).

T-cell NHL. Since the first reported detection of EBV in T-cell NHL (Jones *et al*. 1988), it has become clear that EBV is detectable in T-cell NHL more frequently than in B-cell NHL (Pallesen *et al*. 1994; IARC 1997). Sinonasal angiocentric T-cell NHL (so-called lethal midline granuloma) is the T-cell NHL showing the most consistent association with the virus (Harabuchi *et al*. 1990). In these tumours the virus is detectable in virtually all tumour cells, the viral episomes are usually clonal and there is a type II pattern of viral latency (IARC 1997). EBV is also frequently detectable in other T-cell NHL. However, in most of these, the virus is present only in variable proportions of tumour cells (Korbjuhn *et al*. 1993; Pallesen *et al*. 1994). Moreover, a proliferation of EBV-infected B-cells in lymph nodes affected by EBV-negative T-cell NHL has been reported (Hojo *et al*. 1995; Ho *et al*. 1998; Niedobitek *et al*. 2000a). The significance of these observations for the pathogenesis of T-cell NHL remains uncertain.

EBV-associated carcinomas

Nasopharyngeal carcinoma. NPCs are endemic in South-east Asia, northern Africa, and some other regions, while they occur rarely in western Europe and North America (Niedobitek, 2000). Non-keratinizing undifferentiated NPC, usually associated with a prominent admixture of tumour-infiltrating lymphocytes, represents the most common histological type while

conventional squamous cell NPC are less common (Niedobitek *et al*. 1996). Independently of the geographical origin of the patients, undifferentiated NPCs are always EBV-positive and harbour monoclonal viral episomes (Figure 1g) (Raab-Traub & Flynn 1986; Niedobitek *et al*. 1996). Expression of LMP1 can be detected at the mRNA or protein level in most undifferentiated NPCs (Figure 1h) (IARC 1997). LMP2A expression in NPC cells is detectable at the transcriptional level but the protein is usually undetectable by immunohistochemical techniques (Niedobitek, 2000). Expression of another EBV latent gene, BARF1, with possible transforming functions has also been detected in NPCs (Decaussin *et al*. 2000). The association of undifferentiated NPC with EBV has been well documented. By contrast, an association of the virus with squamous cell NPC is controversial (IARC 1997). This issue has been resolved by a study demonstrating that EBV is present in all squamous cell NPCs from an endemic area, while the virus is detectable only in about 30% of cases from regions with a low NPC incidence (Nicholls *et al*. 1997). This suggests that other factors may be able to replace EBV in the pathogenesis of squamous cell NPC. Smoking and infection with human papillomaviruses are likely to be of importance in this respect (Hording *et al*. 1994; Vaughan *et al*. 1996).

Other carcinomas. Carcinomas showing morphological features similar to undifferentiated NPCs, so-called lymphoepithelial carcinomas, can arise at other sites and these tumours have been extensively studied for an association with EBV. These studies have identified three different groups. Lymphoepithelial carcinomas of the stomach are EBV-associated in approximately 80% of cases regardless of the origin of the patients (Osato & Imai 1996). Lymphoepithelial carcinomas of the salivary glands, the lungs and possibly of the thymus are frequently associated with EBV infection in areas where NPC is endemic whereas similar tumours arising, e.g. in Caucasian patients, appear to be EBV-negative (IARC 1997). Finally, there is a large group of EBV-negative lymphoepithelial carcinomas, including carcinomas of the cervix uteri, the urinary bladder, the skin, and the larynx (IARC 1997). It has to be noted, however, that this statement relies largely on single case reports and studies of small series. Systematic comparative studies are lacking, probably owing to the scarcity of these tumours.

The detection of EBV in gastric lymphoepithelial carcinomas has prompted several groups to study conventional gastric adenocarcinomas, revealing the presence of EBV in 2–16% of cases world-wide (Osato & Imai 1996; IARC 1997). Studies of EBV latent gene expression in gastric carcinomas (lymphoepithelial and adenocarcinomas) have revealed the expression of EBNA1 in the absence of EBNA2 and LMP1 (Osato & Imai 1996). Expression of LMP2A and of BARF1 has been detected at the transcriptional level (Osato & Imai 1996; zur Hausen *et al*. 2000). An association with EBV has also been reported for a proportion of breast carcinomas (Labrecque *et al*. 1995). This finding has been unexpected since studies of medullary carcinoma of the breast, which shares some morphological features with undifferentiated NPC have consistently failed to identify the virus (Niedobitek *et al*. 1991; Kumar & Kumar 1994; Lespagnard *et al*. 1995). Thus, this observation requires confirmation. Finally, EBV has been detected in approximately 35% of hepatocellular carcinomas using Southern blot hybridization (Sugawara *et al*. 1999). Interestingly, analysis of the terminal repeats of the EBV genome revealed monoclonal viral genomes while immunostaining for the EBNA complex suggested that the virus was present only in a proportion of the tumour cells (Sugawara *et al*. 1999). The most surprising finding of this study was the absence of detectable expression of the EBERs suggesting a new type of latency (Sugawara *et al*. 1999).

Other tumours. Unexpectedly, EBV infection has also been detected in leiomyosarcomas, i.e. smooth muscle cell-derived sarcomas, in immunosuppressed patients. Several authors have demonstrated monoclonal or biclonal viral genomes in these tumours. Analysis of viral gene expression has revealed expression of the EBERs and EBNA2 in the absence of LMP1 (Lee *et al*. 1995; McClain *et al*. 1995). Clonal EBV infection has also been detected in inflammatory pseudotumours derived from CD21-positive follicular dendritic reticulum cells (Selves *et al*. 1996).

EBV-associated tumours: specific problems

Role of the immune system

The notion of an antitumour immunosurveillance system is controversial (Prehn 1974). However, there is no doubt regarding the existence of an immunological control of tumour viruses (Prehn 1974). In the case of EBV, this is illustrated by the development of an EBV-specific T-cell immunity in the course of primary infection which permits the establishment of an asymptomatic persistent infection (Rickinson & Kieff 1996). The importance of EBV specific CTLs has been further underlined by the observation of EBV-associated

lymphoproliferative disorders in immunosuppressed transplant patients (PTLD) (IARC 1997). PTLD comprise a spectrum of diseases ranging from polyclonal lymphoproliferations to monoclonal monomorphic PTLDs indistinguishable from malignant lymphomas arising in immunocompentent individuals (Craig *et al*. 1993). The latter notably include not only NHL but also HD cases (Garnier *et al*. 1996). This diversity is accompanied by diverse patterns of EBV latent gene expression ranging from latency III in many polyclonal PTLDs to latencies II or I in monoclonal monomorphic PTLDs (Oudejans *et al*. 1995; Niedobitek *et al*. 1997a). A large body of evidence suggests that the range of lesions encountered in transplant patients may be indicative of a process. According to this model, a relaxation of the EBV-specific immunity allows a polyclonal outgrowth of EBV-infected cells with a latency III pattern of EBV gene expression similar to that seen in infectious mononucleosis. Through the acquisition of additional genetic alterations, e.g. c-myc translocations or p53 mutations, this may eventually progress to frank malignant lymphoma (Delecluse *et al*. 1995; Knowles *et al*. 1995). This idea is supported, e.g. by the observation that polyclonal polymorphic PTLD may show progression to monoclonal monomorphic malignant lymphoma, whereas the reverse does not occur (Wu *et al*. 1996). However, whether those cases which initially present as malignant lymphomas were preceded by a period of subclinical polyclonal proliferation, or represent true *de novo* development of malignant lymphoma, remains uncertain. The importance of immunosuppression in the pathogenesis of PTLD is also underlined by the frequent regression of PTLD lesions in response to a reduction or cessation of immunosuppressive therapy and by recent successful efforts to prevent and treat PTLD using EBV-specific CTLs generated *in vitro* (Starzl *et al*. 1984; Rooney *et al*. 1995; Rooney *et al*. 1998).

Apart from the lymphomas arising in immunocompromised individuals, most EBV-associated tumours develop in patients without systemic immunodeficiencies. Specifically, the immune systems of these patients appear to be quite capable of containing EBV infection as illustrated by the absence of lymphoproliferations characteristic for immunodeficient individuals. This raises the question as to why EBV-associated tumours, such as BL, HD or NPC, are not rejected by the immune system. In the case of BL, an important feature appears to be the absence of those viral antigens which provide CTL target epitopes. In particular, EBNA1, the only known latent protein consistently expressed in BL cells, does not elicit a response by $CD8^+$ CTLs. The reason for this appears to be a Gly-Ala repeat sequence within the EBNA1 protein which blocks ubiquitin-dependent intracellular processing and presentation within the context of MHC class I (Levitskaya *et al*. 1995; Levitskaya *et al*. 1997). Thus, these tumour cells become invisible for EBV-specific CTL. The role of EBNA1-specific $CD4^+$ T-cells in the pathogenesis of BL remains to be clarified (Münz *et al*. 2000). Using immunohistochemistry, isolated cells expressing LMP1 or EBNA2 are detectable in some cases of endemic BL (Niedobitek *et al*. 1995). This illustrates that the phenotypic shift from a type I to a type III latency seen in BL cells *in vitro* may also occur *in vivo*. That these cells do not gain a proliferative advantage over the $EBNA1^+/EBNA2^-/LMP1^-$ tumour cells may well be due to an intact EBV-specific CTL response which eliminates such cells. Interestingly, it has been reported recently that EBNA1 induces a response of $CD4^+$ T-cells which predominatly secrete Th2 cytokines. Since many of these cytokines are known growth factors for EBV-infected B-cells this may contribute to the growth of EBV-associated BL (Steigerwald-Mullen *et al*. 2000).

By contrast, the tumour cells of HD and NPC express LMP1 and LMP2A, antigens known to elicit CTL reponses (Herbst *et al*. 1991; Niedobitek *et al*. 1997). Moreover, HD and NPC are characterized by the presence of numerous lymphoid and other inflammatory cells admixed with the tumour cells which, in the case of HD, vastly outnumber the neoplastic cells (Herbst *et al*. 1993; Niedobitek 2000). In both neoplasms, the tumour cells frequently express CD40, CD70, CD80, and CD95 while the corresponding receptors/ligands (CD40L, CD27, CD28, and CD95L, respectively) are detectable in admixed lymphocytes (Agathanggelou *et al*. 1995; Poppema *et al*. 1998; Abdulkarim *et al*. 2000). This indicates that there may be functional interactions between the neoplastic cells and the tumour-infiltrating lymphocytes in HD and NPC. Moreover, expression of CD40, CD70, and CD80 in nonimmunogenic tumour cells can induce antitumour CTL responses in animal experiments (Nieland *et al*. 1998). In vitro, the tumour cells of NPC and HD are capable of antigen-processing and are recognized and lysed by HLA class I-restricted virus-specific CTLs (Sing *et al*. 1997; Khanna *et al*. 1998; Lee *et al*. 1998). Moreover, NPCs are susceptible to Fas-mediated lysis although this may be inhibited by CD40 signalling (Sbih-Lammali *et al*. 1999). Thus, these observations raise the question as to why HD and NPC can grow *in vivo* in the face of an otherwise functioning EBV-specific immune reponse. Finding an answer to this question is particularly important in view of recent attempts to develop immunotherapeutic approaches to

the treatment of HD and NPC which are based on EBV-specific CTLs (Ambinder *et al*. 1996).

The T-cells surrounding the HRS cells are mainly activated $CD4^+$ cells with a Th2 phenotype (Poppema *et al*. 1998). Several studies have indicated that HRS cells, through the production of cytokines and chemokines, may contribute to and modulate the accumulation of lymphocytes in affected tissues (Poppema *et al*. 1998). HRS cells express the thymus and activation regulated chemokine (TARC) which selectively recruits CCR4-positive Th2 cells (Imai *et al*. 1999; van den Berg *et al*. 1999). Furthermore, it appears that EBV-specific CTL are absent from lymph nodes affected by EBV-positive but not those affected by EBV-negative HD (Frisan *et al*. 1995). This effect may be partially mediated by interleukin (IL)-10 which is strongly expressed in EBV-positive HRS cells and to a lesser extent in EBV-negative HRS cells (Herbst *et al*. 1996; Bejarano & Masucci 1998). Moreover, IL-6 expression has been observed preferentially in EBV-positive HRS cells (Herbst *et al*. 1997). Thus, although HRS cells are effective antigen presenting cells susceptible to CTL lysis, they seem to be able to modulate actively their microenvironment resulting in an ineffective immune response.

In comparison to HD, the interactions between tumour cells and tumour-infiltrating lymphocytes in NPC are less well understood. Like HRS cells, NPC cells appear to be able to secrete cytokines. Variable expression of IL1α, IL1β, TNFα, and IL-8 has been reported in some NPC cell lines, but a consistent pattern is yet to emerge (Busson *et al*. 1987; Mahe *et al*. 1992). Moreover, there is only very limited, and in parts conflicting, information available regarding the cytokine expression patterns of NPCs *in vivo*. Huang *et al*. have demonstrated IL-1 expression in NPC tumour cells with a number of other cytokines expressed in the lymphoid stroma (Huang *et al*. 1999). IL-10 expression has been reported and has even been suggested to be a marker of poor prognosis in NPCs (Yao *et al*. 1997; Fujieda *et al*. 1999). However, studying a large series of NPCs by *in situ* hybridization, we could not confirm this observation (Beck *et al*. 2001). Using *in situ* hybridization, IL-10-specific transcripts were only detectable in some tumour-infiltrating lymphocytes but not in the tumour cell population. IL-6 and IL-8 expression was detectable only in exceptional cases and then only in a small fraction of the tumour cells raising questions as to the significance of this finding (Beck *et al*. 2001). TARC expression was undetectable in NPCs (Beck *et al*. 2001). Elevated serum levels of TGFβ have been reported in NPC patients but the cellular source of this cytokine is presently uncertain (Xu *et al*. 1999). Yet another mechanism for immune escape of NPC cells has been suggested by the demonstration that the LMP1 gene in NPCs may be mutated, rendering it less immunogenic in animal experiments (Trivedi *et al*. 1994; Hu *et al*. 2000). No such data are available for HD.

In summary, albeit of different lineage, the tumour cells of HD and NPC share some interesting features. In both diseases, the neoplastic cells bear all the hallmarks necessary for antigen presentation and are susceptible to EBV-specific CTL killing *in vitro*. They share striking phenotypic similarities which allow them to communicate with the tumour-infiltrating lymphocytes. Nevertheless, in both tumours an effective immunological control of the tumour cells is not apparent. In HD, HRS cells are capable of secreting an array of cytokines and chemokines which may modulate the immunological response in the microenvironment of the neoplastic cells and this may allow the tumour cells to escape immune control. In NPC, similar mechanisms may be at work, although consistent patterns of cytokine and chemokine expression have not yet been identified.

Another scenario is encountered in rare cases of high-grade B-cell lymphoma occurring in association with chronic inflammatory processes, e.g. pyothorax or osteomyelitis. In such cases, one may encounter latency forms II or even III suggesting a defect of EBV-specific immunity (Copie-Bergman *et al*. 1997; Fukayama *et al*. 1993; Sasajima *et al*. 1993; Martin *et al*. 1994). However, the affected patients do not display systemic immunodeficiencies and the lymphomas develop at the site of inflammation. The latter observations suggest that in the context of chronic suppurative inflammation, a local breakdown of EBV-specific immunity may occur. Thus, a loss of immunological control of EBV infection at different levels – systemic, local or in the immediate microenvironment of the tumour cells – may contribute to the development of EBV-associated malignancies.

The timing of EBV infection in the pathogenesis of EBV-associated tumours

In most EBV-associated tumours, the virus is detectable in virtually all tumour cells. Moreover, the viral genomes are usually present as monoclonal episomes, i.e. they are derived from a single infection event (Raab-Traub & Flynn 1986). This has been shown, e.g. for BL, HD, and NPC (Raab-Traub & Flynn 1986; Anagnostopoulos *et al*. 1989). These findings are taken to imply that EBV infection takes place before expansion of the neoplastic clone. Thus, it appears that EBV infection in these tumours occurs early enough to play a part in the

carcinogenic process. Nevertheless, in most virus-associated tumours the question as to when exactly in the carcinogenic process EBV infection occurs remains controversial.

EBV infection is detectable in virtually all cases of undifferentiated NPC regardless of the geographical origin of the patients (Niedobitek, 2000). Thus, EBV infection appears to be a rate-limiting step in the pathogenesis of NPC. The viral genomes are present in all tumour cells and are of clonal origin suggesting that infection occurs early in the neoplastic process (Raab-Traub & Flynn 1986; Niedobitek, 2000). This idea is confirmed by the detection of EBV in *in situ* NPC lesions, which are presumed precursor lesions of invasive NPC (Pathmanathan *et al*. 1995). Moreover, clonal progression from mild dysplasia to invasive carcinoma has been reported (Jiang & Yao 1996). However, EBV has not been detected in normal nasopharyngeal epithelial cells in a high-risk population nor in normal nasopharyngeal mucosa adjacent to EBV-positive nasopharyngeal carcinoma (Sam *et al*. 1993). This would seem to suggest that EBV infection of epithelial cells may not be the first step in NPC pathogenesis. This notion is supported by two observations. Chromosome 3p deletions are detectable in 80–100% of nasopharyngeal carcinoma cases and thus appear to be almost as common as EBV infection in these tumours (Chan *et al*. 2000). Interestingly, 3p deletions are also equally common in EBV-positive dysplatic lesions of the nasopharynx and in EBV-negative normal nasopharyngeal mucosa in individuals from a high-risk population (Chan *et al*. 2000). This would seem to suggest that 3p deletions precede EBV infection in the pathogenesis of NPC. Moreover, stable EBV infection of CD21-transfected epithelial cells has been shown to require an undifferentiated phenotype (Knox *et al*. 1996). Thus, it appears that EBV infection is not the first step in the pathogenesis of NPC but occurs before the initiation of invasive growth.

Malignant lymphomas lack well defined preneoplastic stages and thus determination of the timing of EBV infection has to rely on indirect evidence. Endemic BLs are virtually always EBV-positive, while the virus is detectable only in up to 30% of sporadic BLs (IARC 1997). However, all cases harbour a translocation juxtaposing the c-*myc* gene to one of the immunoglobulin gene loci. In endemic BL, this translocation is reminiscent of a V(D)J recombination event, while it resembles a switch recombination in sporadic BL (Haluska *et al*. 1986; Vanasse *et al*. 1999). Early studies have suggested that *c-myc* translocations occur in EBV-infected B-cells and that a malaria-induced B-cell proliferation increases the chance of such transloactions occurring (Klein 1979). The assumption that *c-myc* translocations, at least in endemic BL, are the result of an accident during immunoglobulin gene rearrangements in immature B-cells on the other hand has raised the possibility that *c-myc* translocation may precede EBV infection (Lenoir & Bornkamm 1987). Molecular studies have indicated that EBV infection is a late event in endemic BL occurring after c-*myc* translocation (Gutierrez *et al*. 1997). V(D)J recombination of immunoglobulin and T-cell receptor genes physiologically occurs in immature B-and T-cells and is initiated by two recombination activating genes, RAG1 and RAG2 (Oettinger *et al*. 1990). Interestingly, expression of the RAGs has been reported in EBV-positive endemic but not in EBV-negative sporadic BL cell lines (Kuhn-Hallek *et al*. 1995; Srinivas & Sixbey 1995). Moreover, RAG expression became detectable in sporadic BL cell lines following EBV infection, an effect apparently mediated by EBNA1 and suppressed by LMP1 (Kuhn-Hallek *et al*. 1995; Srinivas & Sixbey 1995). These findings suggest that EBV infection in the context of a latency I may be able to induce RAG expression and thus offer a possible link between EBV infection and the occurrence of c-*myc* translocations in endemic BL. However, using *in situ* hybridization, we have been unable to detect RAG expression in EBV-associated endemic BLs (Meru *et al*. 2001). It appears therefore that re-induction of RAG expression by EBV is not an important mechanism for the pathogenesis of EBV-associated endemic BL and in particular is not relevant for the development of c-*myc* translocations. Taken together, the balance of this evidence suggests that *c-myc* translocation precedes EBV infection in the pathogenesis of EBV-associated endemic BL. However, that this issue is by no means settled is illustrated by reports suggesting that genetic alterations, including oncogene translocations, may be the result of the somatic hypermutation process in germinal centres (Goossens *et al*. 1998). All BL cases analysed thus far display somatically mutated V region genes indicating that they have participated in germinal centre reactions. Moreover, two BL cases have been described in which the $V_\kappa J_\kappa$ region and the C_κ locus both showed evidence of somatic hypermutation although being separated by c-*myc* translocations (Goossens *et al*. 1998). Thus it is possible that the *c-myc* translocation in BL may occur as a by-product during expansion of EBV-positive B-cells in germinal centre reactions (Niedobitek *et al*. 1992; Goossens *et al*. 1998).

In most HD cases the neoplastic cells are derived from B-cells as demonstrated by the detection of clonal immunoglobulin gene rearrangements in the HRS cells,

and by sequence analysis of the immunoglobulin V region genes revealing evidence of somatic hypermutation (Kanzler *et al*. 1996). Moreover, in some cases, stop codons have been detected in these genes rendering them nonfunctional (Kanzler *et al*. 1996). Thus, it has been argued that HD is a neoplasm derived from 'crippled' germinal centre B-cells which have been rescued from apoptotic cell death by a transforming event (Kanzler *et al*. 1996). Possibly, survival signals in these cells may be delivered by the CD40-like function of LMP1 and/or the B-cell receptor-like signalling activity of LMP2A (Caldwell *et al*. 1998; Kilger *et al*. 1998). This scenario would require EBV infection occurring prior to the 'crippling' event. Support for this idea has come from reports describing the expression of LMP1 in germinal centre B-cells (Araujo *et al*. 1999; Babcock *et al*. 2000).

The observation that in a proportion of T-cell lymphomas EBV is present only in a small proportion of tumour cells has added yet another level of complexity to this discussion (Pallesen *et al*. 1994). It has to be mentioned in this context, however, that at least some cases of T-cell NHL are characterized by an LCL-like background proliferation of EBV-infected B-cells while the neoplastic T-cells are EBV-negative (Hojo *et al*. 1995; Ho *et al*. 1998; Niedobitek *et al*. 2000a). Nevertheless, in some studies of T-cell NHL, the presence of the virus in a proportion of the neoplastic cells has been confirmed by double labelling experiments (Korbjuhn *et al*. 1993). Moreover, similar observations have been reported for some cases of B-cell chronic lymphocytic leukaemia and other B-cell lymphomas (Hummel *et al*. 1995). Thus, EBV infection of a proportion of neoplastic cells appears to occur in some lymphomas while, interestingly, it has not yet been convincingly shown for carcinomas, HD and other virus-associated tumours. This phenomenon is generally believed to be the result of a secondary infection of the neoplastic cells by EBV (Niedobitek 1995). This explanation is supported by a case report demonstrating EBV infection of a metastatic lymph node deposit of an EBV-negative primary intestinal B-cell lymphoma (Ilyas *et al*. 1995). In this scenario, EBV infection of an already established malignancy may confer a growth advantage to the infected cells leading eventually to the replacement of the EBV-negative population. Thus, it is conceivable that superinfection of a primarily EBV-negative tumour with EBV may give rise to an EBV-positive tumour with monoclonal viral genomes present in all tumour cells. Alternatively, loss of the viral genome from neoplastic cells has to be considered. This explanation is supported by a case report of an EBV-positive HD case relapsing as EBV-negative HD (Delecluse *et al*. 1997). In addition, viral genomes may be lost from BL and NPC cells cultured *in vitro* (Lin *et al*. 1993; Shimizu *et al*. 1994; Cheung *et al*. 1999; Ruf *et al*. 1999). However, at least in the case of BL this appears to coincide with a loss of tumorigenicity (Shimizu *et al*. 1994). Interestingly, reinfection with EBV, but not transfection with EBNA1, has been demonstrated to reestablish the malignant phenotype (Ruf *et al*. 1999). Thus, the evidence indicating that loss of viral genomes may occur in EBV-associated tumours is mainly derived from *in vitro* studies. Moreover, it has been demonstrated only for those tumours in which partial infection of the neoplastic cells *in vivo* has not been reported as yet.

EBV – a human carcinogen?

EBV is a ubiquitous herpesvirus which establishes a lifelong persistent infection in over 90% of adults worldwide. This persistent infection is usually asymptomatic. It has been known for almost 40 years that EBV is associated with two human malignancies, i.e. Burkitt's lymphoma and nasopharyngeal carcinoma (Epstein *et al*. 1964; Wolf *et al*. 1973). In addition, EBV has been identified in a large number of other tumours from different cell lineages (IARC 1997). Thus, EBV is one of the more common potential human carcinogens. This makes an assessment of the role of EBV in the pathogenesis of these diverse cancers important. Based on its association with Burkitt's lymphoma, Hodgkin's disease, lymphoproliferations in immunosuppressed individuals, sinonasal angiocentric T-cell lymphoma, and nasopharyngeal carcinoma, EBV has been classified as a group I carcinogen by the IARC (IARC 1997). There is now ample evidence for a transforming function of EBV *in vitro*. Most importantly, LMP1 has been identified as a viral oncogene with the ability to transform rodent fibroblasts and with functions similar to those of a TNF receptor family member (Wang *et al*. 1985; Baichwal & Sugden 1988; Gires *et al*. 1997). Nevertheless, LMP1 alone is not sufficient to transform B-cells *in vitro* but requires the cooperation of several other viral latent proteins. The effects of EBV infection of B-cells in many ways parallel those of antigen activation and amount to immortalization. While this is an important step in carcinogenesis, it is by no means sufficient.

The role of the virus in the pathogenesis of human neoplasms *in vivo* remains debated and much of the evidence supporting such a role is circumstantial. In most virus-associated tumours, the EBV genomes appear to be of monoclonal origin (Raab-Traub & Flynn 1986). This would seem to point to an infection

before expansion of the malignant cell clone. However, studies of various tumours now suggest that while occurring early, EBV infection may not be the first event in the carcinogenic process. Thus, in some tumours, EBV is detectable only in a proportion of the neoplastic cells. In others, such as BL and NPC, careful genetic studies have demonstrated that characteristic chromosomal alterations occur prior to EBV infection. Whilst this does not exclude a contribution of EBV to the pathogenesis of these tumours it is obvious that further studies are required to define the role of EBV. The expression of viral antigens, notably of LMP1, in many virus-associated tumours has been taken as further evidence to support a role for EBV in the pathogenesis of human tumours. However, the patterns of viral latent protein expression in human tumours are variable, suggesting that the contribution of EBV to different carcinogenic processes may also vary. Moreover, the majority of viral proteins which are required for the immortalization of B-cells *in vitro* are not expressed in EBV-associated tumours. Also, LMP1 and other viral proteins are clearly detectable in situations not associated with malignancy, notably infectious mononucleosis. The strongest support for an oncogenic role of EBV has come from the observation of EBV-associated lymphoproliferations in immunosuppressed patients (IARC 1997). However, although these lymphoproliferations are frequently fatal, they often do not meet strict criteria of neoplasia. Thus, rather than supporting an oncogenic role of EBV, this observation highlights the importance of the immune system in controlling EBV infection.

In summary, the picture which has emerged after almost 40 years of EBV research is complex. While a role for EBV in the pathogenesis of certain human tumours remains likely, the precise contribution of the virus remains to be defined.

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